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PRINCIPAL INVESTIGATOR: Shaomeng Wang, Ph.D.

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, MI 48109-1274

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Shaomeng Wang, Ph.D.

E-mail: shaomeng@umich.edu

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14. ABSTRACT
Overexpression of Bcl-2 has been observed in 70% of breast carcinomas and the expression levels of Bcl-2 proteins correlate with resistance to a wide spectrum of chemotherapeutic drugs and radiation therapy. In this IDEA grant, we propose an effective structure-based approach to discover small molecule inhibitors of Bcl-2 through structure-based 3D-database search over large chemical databases containing >500,000 structurally diverse, non-peptide, drug-like synthetic compounds or natural products. Using this powerful approach, we have discovered 10 classes of structurally diverse, non-peptidic, drug-like, small-molecule inhibitors of Bcl-2. Our studies also showed that the most promising small-molecule inhibitors of Bcl-2 we have discovered potently bind to Bcl-2 protein, inhibit cell growth and induce apoptosis in breast cancer cells with high levels of Bcl-2 proteins and display good selectivity in cancer cells with low levels of Bcl-2 proteins. Furthermore, our most potent small-molecule inhibitor of Bcl-2 inhibits tumor growth in animal models of human breast cancer. Our results have demonstrated that potent small-molecule inhibitors of Bcl-2 may have a great therapeutic potential for the treatment of human breast cancer by overcoming apoptosis resistance of breast cancer cells.

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Introduction: Bcl-2 is the founding member of the Bcl-2 family proteins and potently inhibits apoptosis in cells. As a potent anti-apoptotic molecule, Bcl-2 contributes to cancer cell progression by preventing normal cell turnover caused by physiological cell death mechanisms. Overexpression of Bcl-2 has been observed in 70% of breast carcinomas. The expression levels of Bcl-2 proteins correlate with resistance to a wide spectrum of chemotherapeutic drugs and radiation therapy. The experimental three-dimensional (3D) structure of Bcl-2 showed that Bcl-2 has a surface binding pocket into which pro-apoptotic proteins such as Bid, Bim and Bad bind. This pocket is essential for the anti-apoptotic function of Bcl-2 since mutations at this site abolished Bcl-2 biological function. Therefore, we hypothesize that non-peptide, drug-like, cell permeable small molecules that bind to this surface pocket of Bcl-2 will block the anti-apoptotic function of Bcl-2 and may restore the normal apoptotic process in cancer cells with Bcl-2 protein overexpression and make these cancer cells more susceptible to conventional chemotherapy or radiation therapy. Designing of small molecule inhibitors targeting Bcl-2 at this crucial binding site represents an attractive approach for the development of a novel therapy for the treatment of breast cancer with Bcl-2 protein overexpression.

In this IDEA grant, we propose an effective structure-based approach to discover small molecules that bind to the Bcl-2 binding pocket. Specifically, we propose to perform structure-based 3D-database search over large chemical databases containing >500,000 structurally diverse, non-peptide, drug-like synthetic compounds or natural products to identify small molecule candidates that can effectively interact with the Bcl-2 binding pocket. Most promising candidate molecules are then tested in appropriate binding and cellular assays to confirm their activity, specificity and mechanism. For the best Bcl-2 inhibitors identified from this project, they will be further evaluated for their anti-cancer activity *in vivo* and their therapeutic potential for the treatment of human breast cancer with high levels of Bcl-2 protein.

Discovery of novel, non-peptidic, cell permeable Bcl-2 small molecule inhibitors represents the first but very exciting step toward the development of a novel cancer therapy targeted at Bcl-2. The success of this project will pave the way for the development of a small molecule drug through modulation of the Bcl-2 function for the treatment of breast and many other forms of cancers with Bcl-2 overexpression, either alone or in combination with conventional chemotherapeutic drugs or radiation therapy.

Body of the report:

Task 1. Molecular modeling, structure-based database searching, and computational docking (**1-30 months**).

Task 1.1. Extensive molecular dynamics simulation of Bcl-2 through molecular dynamics simulations.

This task was completed and the report was submitted last year.

Task 1.2. Structure-based 3D-database searching on four 3D-databases containing more than 650,000 small organic compounds and natural products to identify most promising small molecule inhibitors that effectively interact with the Bcl-2 surface-binding pocket. (**1-30 months**).

This task has been essentially completed and the results were reported last year. However, since we have recently built another large chemical database consisting of 200,000 druglike, organic small-molecules we obtained from ASINEX. We have performed a structure-based database searching with a goal to identify additional structurally diverse small-molecule inhibitors that are entirely different from those inhibitors we have previously discovered. We have ordered chemical samples of **50** candidate small-molecule inhibitors that have the highest ranks based upon the scores. These candidate compounds will be tested in our established Bcl-2 binding assay to determine their binding affinities to Bcl-2. Most potent confirmed small-molecule inhibitors of Bcl-2 will be further evaluated

Task 2. *In vitro* biological confirmation of potential Bcl-2 inhibitors and mechanism investigations (**3-30 months**).

Task 2.1. Testing of potential small molecule inhibitors of Bcl-2 using an *in vitro* fluorescence polarization (FP) based binding assay.

Since many human breast cancer cell lines overexpress not only Bcl-2 but also Bcl-xL and Mcl-1, Bcl-xL and Mcl-1 are important Bcl-2 members that may play key roles for the resistance of human breast cancer cells to current therapeutic agents. Thus, we have now developed assays for other Bcl-2 members, including Bcl-xL and Mcl-1 and we have evaluated our most potent

small-molecule inhibitors for their binding to Bcl-xL and Mcl-1. Using these assays, it was determined that (-)-gossypol binds to Bcl-xL with a K_i value of 710 nM. In addition, (-)-gossypol also binds to Mcl-1 with a K_i value of 160 nM. Therefore, our results indicate that (-)-gossypol is a potent pan-Bcl-2 inhibitor. Since many human breast cancer cells have very high levels of not only Bcl-2 but also Bcl-xL and Mcl-1, a potent pan-Bcl-2 inhibitor such (-)-gossypol may be particularly effective for the treatment of human breast cancer.

Our discovery that (-)-gossypol is a potent inhibitor against not only Bcl-2, but also Bcl-xL and Mcl-1 indicates that it is possible to design potent, small-molecule inhibitors against multiple anti-apoptotic Bcl-2 members. Such agents may be more effective than inhibitors that only target Bcl-2 alone.

Task 2.2. Testing the activity of small molecule inhibitors of Bcl-2 in human breast cancer cells.

This task has been essentially completed and was reported last year.

Task 3. *In vivo* testing of 2-3 most promising lead compounds (**24-36 months**).

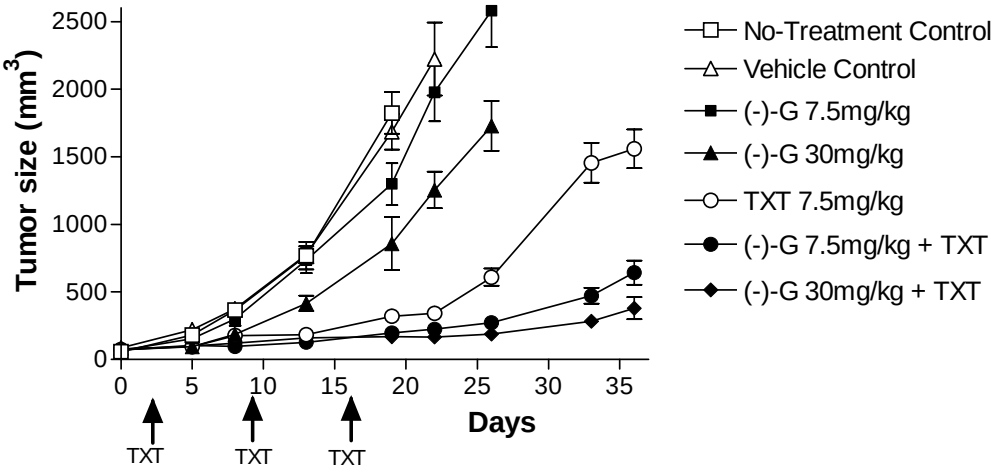
The in vivo studies for (-)-gossypol have been performed. The results are briefly summarized below.

Based upon our *in vitro* data, (-)-gossypol potently inhibits cancer cell growth and induce apoptosis in cancer cells with high levels of Bcl-2 and Bcl-xL proteins. Importantly, (-)-gossypol has a good selectivity to normal cells. Hence, (-)-gossypol may represent a potent and highly promising small-molecule inhibitor of Bcl-2 and Bcl-xL and warrants further investigation for its therapeutic potential for the treatment of human breast cancer. We have therefore synthesized grams of quantity of (-)-gossypol and carried out *in vivo* studies to test its anti-tumor activity in animal model of human breast cancer using the MDA-MB-231 xenograft model in nude mice. The results are summarized in **Figure 1**. Each group of mice consisted of at least 5 mice and 10 tumors. As can be seen, (-)-gossypol has a dose-dependent, potent activity in inhibition of tumor growth. At 30 mg/kg (p.o. daily dose for 4 weeks), (-)-gossypol achieved more than 50% of tumor growth inhibition. Of note, (-)-gossypol is an orally available agent, a major advantage in the development of a novel anti-cancer drug.

Our hypothesis predicted that a potent small-molecule inhibitor may achieve a greater anti-tumor activity when used in combination with chemotherapeutic agents. To test this

hypothesis, we have tested the anti-tumor activity of (-)-gossypol in combination with a commonly used anti-cancer agent Taxotere (TXT). The results are shown in **Figure 1**.

Figure 1. *In vivo* antitumor activity of (-)-gossypol ((-)-G) in the MDA-MB-231 (2LMP) xenograft model in nude mice, alone or in combination with taxotere (TXT).



As can be seen, although TXT (7.5 mg/kg, *i.v.* weekly for three weeks) has a good anti-tumor activity, the combination with either 7.5 mg/kg or 30 mg/kg of (-)-gossypol achieved a much greater anti-tumor activity than either agent alone. Overall, greater than 95% of tumor growth inhibition was achieved when (-)-gossypol was combined with TXT. The results are also highly statistically significant ($p < 0.001$).

Taken together, our data showed that (-)-gossypol achieves a good anti-tumor activity *in vivo* in inhibition of tumor growth when used as a single agent. Importantly (-)-gossypol achieves a much greater anti-tumor activity *in vivo* when used in combination with TXT. These data indicate that (-)-gossypol may have great therapeutic potential to be developed as a novel class of anti-cancer drug for the treatment of human breast cancer and other types of cancer.

Task 4. Preparing scientific publications (**6-36 months**).

The following manuscripts have been accepted for publications or in preparation. The DOD grant support is acknowledged in these manuscripts.

1. Guoping Wang, Zaneta Nikolovska-Coleska, Chao-Yie Yang, Renxiao Wang, Guozhi Tang, Sanjeev Shangary, Su Qiu, Wei Gao, and Shaomeng Wang, Structure-Based Design of Potent Small-Molecule Inhibitors of Anti-apoptotic Bcl-2 Proteins, (manuscript in preparation and to be submitted to *Journal of Medicinal Chemistry*).

2. Zaneta Nikolovska-Coleska, Dajun Yang, York Tomita[3], Peter P. Roller, Shaomeng Wang, Structure Based Discovery Of Gossypol as an Inhibitor Of Bcl-2 Family Proteins and Caracterization of Gossypol Enantiomers, (manuscript in preparation and to be submitted to *Journal of Medicinal Chemistry*).

3. Dajun Yang, Manchao Zhang, Jianyong Chen, Zaneta Nikolovska-Coleska, Liang Xu, Marc E. Lippman, York Tomita[3], Peter P. Roller, Shaomeng Wang, Molecular Mechanism and Pre-Clinical Testing Of (-)-Gossypol as a Potent Inhibitor of Bcl-2 and Bcl-xL for Apoptosis Targeted Anti-Cancer Therapy, manuscript in preparation and to be submitted to *Cancer Research.*

4. Oliver CL, Bauer JA, Wolter KG, Ubell ML, Narayan A, O'Connell KM, Fisher SG, Wang S, Wu X, Ji M, Carey TE, Bradford CR. In vitro Effects of the BH3 Mimetic, (–)-Gossypol, on Head and Neck Squamous Cell Carcinoma Cells, Clin Cancer Res. **2005**, Aug 1;11(15):5659.

5. Bauer JA, Trask DK, Kumar B, Los G, Castro J, Lee JS, Chen J, Wang S, Bradford CR, Carey TE. Reversal of cisplatin resistance with a BH3 mimetic, (-)-gossypol, in head and neck cancer cells: role of wild-type p53 and Bcl-xL. *Mol Cancer Ther.* **2005**, Jul;4(7):1096-104.

6. L. Xu, D. Yang, S. Wang, W. Tang, M. Liu, M. Davis, J. Chen, J. M. Rae, T. Lawrence, and M. E. Lippman, (-)-Gossypol enhances response to radiation therapy and results in tumor regression of human prostate cancer, Mol. Cancer Ther., February 1, **2005**; 4(2): 197 - 205.

7. Mohammad RM, Wang S, Aboukameel A, Chen B, Wu X, Chen J, Al-Katib A.

Preclinical studies of a nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-X(L) [(-)-gossypol] against diffuse large cell lymphoma. *Mol Cancer Ther.* **2005,** Jan;4(1):13-21.

Key Research Accomplishments:

(1). We have discovered and characterized gossypol and its enantiomers as potent small-molecule inhibitors of Bcl-2 and Bcl-xL.
(2). We demonstrated that (-)-gossypol has potent activity in human breast cancer cells and other cancer cells with high levels of Bcl-2 and Bcl-xL proteins and show good selectivity in normal cells with low levels of Bcl-2/Bcl-xL proteins.
(3). We showed that (-)-gossypol achieves a significant anti-tumor activity in vivo in inhibition of tumor growth in the MDA-MB-231 xenograft model and has a much greater anti-tumor activity when used in combination with taxotere than either agent alone.
(4). Based upon our discovery, (-)-gossypol ic currently in Phase I-II human clinical trials as a novel +agent for the treatment of human breast cancer and other types of cancer in which Bcl-2/Bcl-xL proteins are highly overexpressed and traditional therapies have failed. If successful, our research will benefit thousands of cancer patients in the near future.

Reportable Outcomes:

1. Zaneta Nikolovska-Coleska, Dajun Yang, York Tomita[3], Peter P. Roller, Shaomeng Wang, Structure Based Discovery Of Gossypol as an Inhibitor Of Bcl-2 Family Proteins and Caracterization of Gossypol Enantiomers, (manuscript in preparation and to be submitted to *Journal of Medicinal Chemistry*).

2. Guoping Wang, Zaneta Nikolovska-Coleska, Chao-Yie Yang, Renxiao Wang, Guozhi Tang, Sanjeev Shangary, Su Qiu, Wei Gao, and Shaomeng Wang, Structure-Based Design of Potent Small-Molecule Inhibitors of Anti-apoptotic Bcl-2 Proteins, (manuscript in preparation and to be submitted to *Journal of Medicinal Chemistry*).

3. Dajun Yang, Manchao Zhang, Jianyong Chen, Zaneta Nikolovska-Coleska, Liang Xu, Marc E. Lippman, York Tomita[3], Peter P. Roller, Shaomeng Wang, Molecular Mechanism and Pre-Clinical Testing Of (-)-Gossypol as a Potent Inhibitor of Bcl-2 and Bcl-xL for Apoptosis Targeted Anti-Cancer Therapy, manuscript in preparation and to be submitted to *Cancer Research.*

5. Oliver CL, Bauer JA, Wolter KG, Ubell ML, Narayan A, O'Connell KM, Fisher SG, Wang S, Wu X, Ji M, Carey TE, Bradford CR. In vitro Effects of the BH3 Mimetic, (–)-Gossypol, on Head and Neck Squamous Cell Carcinoma Cells, Clin Cancer Res. **2005**, Aug 1;11(15):5659.

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8. Mohammad RM, Wang S, Aboukameel A, Chen B, Wu X, Chen J, Al-Katib A. Preclinical studies of a nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-X(L) [(-)-gossypol] against diffuse large cell lymphoma. *Mol Cancer Ther.* **2005,** Jan;4(1):13-21.

9. An invention has been filed with University of Michigan on gossypol and its analogues as a new class of anti-cancer agents.

Conclusions:

Although this Award was only received in July, 2003 due to the transfer of the grant from Georgetown University to University of Michigan, we have essentially accomplished all the goals and aims we have had in our original proposal. More than 10 classes of structurally diverse, non-peptidic, drug-like, small-molecule inhibitors of Bcl-2 have been successfully identified. Using the small-molecule inhibitors of Bcl-2 we have discovered, 5 manuscripts have been published. Three more manuscripts are being submitted for publication. An invention disclosure has been filed with University of Michigan. In summary, this has become a highly productive project. Among those small-molecule inhibitors we have discovered, (-)-gossypol, is a potent Bcl-2/Bcl-xL inhibitor and represents a highly promising anti-cancer agent. Based upon our results and discovery, Phase I-II clinical trials are being performed to evaluate (-)-gossypol as a new anti-cancer agent for the treatment of human breast and other types of cancer.